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PCT APPLICATION NUMBER: *PCT/US00/08767*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

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Certifying Officer

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Cell patterning on glass and polymeric substrates provisional patent

Introduction:

Polymeric and glass surfaces in their native structures have been used as cellular growth substrates for decades. Differing techniques have been utilized to adjust the surface chemistry of these materials to make them more attractive for cell adhesion including: adsorption of cell adhesion molecules, sulfonisation of the material[17], co-polymer blends of extracellular matrix protein fragments such as RGD[13], and chemical oxidation (using solution chemistry) of the surface for further chemical modification (using solution chemistry)[8] such as silanes[14] or thiols[15].

In addition to adjusting the surface of these substrates to render them more attractive for cellular adhesion, techniques have been developed to render the surfaces repulsive for cellular adhesion. The most utilized molecule for cell repulsion is poly(ethylene glycol) (PEG). PEG can be attached to polymeric and glass substrates in many ways. This can include, but is not limited to: chemically activating the substrate to react with a poly(ethylene imide)-PEG molecule[3], aminating an activated surface and reacting it with bifunctional electrophilic molecules such as PEG-epoxide [2,6,17,18,11,16], and also PEG-styrene co-polymer blends[1,9].

The techniques mentioned so far will lead to homo-monolayers, containing one of the cell attractive or cell repulsive moieties. A combination of the above technologies can logically lead to the creation of hetero-monolayers. When the positioning of these cell adhesive and cell repulsive cues can be controlled to a high degree, cells can become patterned on the substrate of choice. Cell patterning has been achieved on glass and metalized glass substrates utilizing silanes[14] and thiols[15] respectively. These methods are successful in selective localization of cells using a multi-step, equipment intensive process, and/or irreproducible techniques such as deep ultraviolet ablation of molecules and/or printing by mechanical stamping.

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR §1.53(b)(2).

Docket No. 99,376	Type a plus sign (+) inside this box:	+

INVENTOR(S)/APPLICANTS(S)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (City and either state or foreign country)
Adams	Terri		Pittsburgh, PA
Kapur	Ravi		Pittsburgh, PA

TITLE OF THE INVENTION (280 character maximum)

Cell patterning on glass and polymeric substrates

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS (check all that apply)

X Specification Number of Pages 9 ☒ Small Entity Statement
☒ Drawing(s) Number of Sheets 2 ☐ Other (specify):

METHOD OF PAYMENT FOR THIS PROVISIONAL APPLICATION FOR PATENT

XX A check or money order is enclosed to cover the Provisional Filing Fee. The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: **13-2490.**	PROVISIONAL APPLICATION FOR PATENT FILING FEE AMOUNT ($)	75.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government
__X__ No _____ Yes, the name of the U.S. Government agency and the Government contract number are: _

Respectfully submitted,
SIGNATURE: [signature] Date: 6/7/99

TYPED or PRINTED NAME David Harper REG. NO. 42,636

Additional inventors are being named on separately numbered sheets attached hereto.

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

(Attorney's Docket No. 99,376)

Applicant or
Patentee: Adams and Kapur

Serial or
Patent No. To be assigned Filed: Herewith

Title: Cell patterning on glass and polymeric substrates

VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS (37 C.F.R. § 1.9(f) AND § 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: **Cellomics, Inc.**

ADDRESS OF CONCERN: **635 William Pitt Way**
Pittsburgh, Pennsylvania 15238

09/701379 . 031805

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 C.F.R. § 121.12, and reproduced in 37 C.F.R. § 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time, or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled **Cell patterning on**

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glass and polymeric substrates
by inventor(s) Terri Adams and Ravi Kapur,

described in

☒ the specification filed herewith.
☐ Application Serial No. filed
☐ Patent No. ______________, issued ____________.

If the rights held by the above identified small business concern are not exclusive, each individual concern or organization having rights in the invention must file verified statements averring to their status as small entities, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR § 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR § 1.9(d), or a nonprofit organization under 37 CFR § 1.9(e).

Each person, concern or organization having any rights to the invention is listed below:

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 99,376)

In re Application of:		)	
	Adams and Kapur	)	Art Unit:
Serial No.:	To be Assigned	)	Examiner:
Filed:	Herewith	)	
For:	Cell patterning on glass and polymeric substrates	)	

Asst. Commissioner for Patents
BOX PROVISIONAL APPLICATION
Washington, D.C. 20231

TRANSMITTAL LETTER

Sir:

1. We are transmitting herewith the attached papers for the above identified new provisional patent application:

   ☒ Patent Specification (9 pages, including cover sheet, claims, and abstract)
   ☒ Drawings (2 sheets)
   ☒ Return Postcard
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2. ☒ A check in the amount of $75.00 is enclosed for the Filing Fee.

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By: [signature]

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing therein, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Lee R. Johnston, Jr.

TITLE IN ORGANIZATION: VP & Chief Financial Officer

ADDRESS OF PERSON SIGNING: 635 William Pitt Way

Pittsburgh, PA 15238

Signature: [signature]

Date: 6/7/99

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In contrast, the present invention provides a novel, affordable, facile, equipment insensitive, reproducible technique of achieving cell patterning on a durable substrate such as glass and plastic.

The present invention can utilize several novel combinations of surface oxidation by oxygen plasma followed by the application of a "stencil" with no feature size restraint and/or formation of a reactive monolayer of organosilane, followed by vapor deposition or solution deposition of any silane or surface reactive cell repulsive or cell attractive moiety around the "stencil", which can be further modified by a backfill with an opposing chemistry utilizing either vapor deposition or solution chemistry. This combination of methods is novel and has many advantages over conventional patterning techniques (see figure 4 for one possible combination of these techniques).

Background:

Oxygen plasma can be achieved with oxygen radio frequency glow discharge. This discharge is accomplished with an instrument that can produce charged particles (electrons and positive ions) that interact with the background gas, (oxygen) to produce free radicals under the time-varying electric field in radio frequency. The sample is placed into a cylindrical reactor, a minimal amount of oxygen gas is introduced, and charged particles are evolved between parallel-plated electrodes resulting in the cleavage of the O_2 bond. After this cleavage, high-energy free radicals can insert themselves into the polymer backbone resulting in the formation of various oxygen moieties, among them are hydroxyl groups. The samples are removed and then reacted with silanes to form the desired self assembling monolayer (SAM).[4,10]

The chemistry of organosilanes is utilized in this invention to produce surfaces with the reactive moiety of choice. In a preferred embodiment, aminosilanes are used. Organosilanes fall into a larger class of molecules, which have the capability of forming self-assembled (SA) films. The general form of this molecule is R_nSiX_{4-n}, where n = 1,2, or 3 and X = Cl, OCH_3, or OC_2H_5. Polymer or glass, can be oxidized so that they present surface hydroxyl groups, organosilanes react with hydroxyl groups to produce covalent Si-O-substrate (siloxane) linkages[14].

The chemistry of 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride), can be used to convert hydroxyl, amine, or thiol groups into good leaving groups that, on reaction with nucleophiles, tresyl chloride will allow stable linkages to be formed between the nucleophile and the initial hydroxyl, amine, or thiol group carrying carbon. In a preferred embodiment, PEG_{5000} is attached to a tresyl group for reaction with surface aminosilane groups. The desired effect is also achievable with surface hydroxyl groups, (which would eliminate any silanizing steps).[5,8]

How this invention differs from present technology

In the patterning method of the present invention, using a "stencil" (mechanical or physical mask, not a printing method) is more advantageous compared to deep UV photolithography, because the materials required to produce the stencil can be made of affordable poly(dimethyl) siloxane (PDMS) or a low energy UV photocurable polymer for instance, as opposed to a costly high energy laser apparatus required for photolithography[14]. The present methods are reproducible when compared to contact printing, because the stencil can be applied to the same spot on each substrate with great accuracy, and there is less opportunity for operator error. There is operator dependence when contact printing due to the subjectivity of applying the stamp to the substrate (force by which the stamp is depressed, amount of solution on the stamp) and so the results will vary[15]. The present method of using a stencil for masking while performing solution

or vapor phase deposition of the cytophobic chemistry is operator independent, thus allowing for a scalable and manufacturable process.

Vapor deposition of the silane or reactive moiety has many benefits. The stencil does not need to make a "solvent tight" seal with the polystyrene to perform its function, and the vapor will not "wick" under a mask as a solvent would. Also, one can use a wider range of silanes because a solvent is not needed. Many silane solvents would dissolve the polymeric substrate and destroy its optical quality. The present method circumvents the use of solvents altogether.

The present invention is not constrained to one particular kind of substrate. The tethering chemistry of the primary monolayer, or the organosilane is such that it reacts with surface hydroxyl groups. These hydroxyl groups can be introduced on the surface of virtually any plastic and glass by low temperature plasma treatment. The secondary tethering chemistry, tresyl chemistry, can react with surface amines, hydroxyl, and thiols making it possible to attach to a wider array of surface chemistry. The instantly disclosed method of cell patterning has a marked advantage over prior thiol chemistry. Previous technology of contact printing with thiols not only introduces operator error, but also requires a thin layer of gold to be evaporated on the tissue culture substrate. Due to the high temperature involved with gold evaporation, most plastics are ruled out. Optical quality is constrained and fluorescence intensity is lowered due to the added layer of gold. In addition to a lower optical quality, there is a high cost associated with gold coating. The methods of the present invention permit cell patterning on an optically clear substrate and give the added option of control over the substrate so that one has the freedom to choose the most superior affordable plastic or glass for optical quality.

The use of a plastic such as polystyrene has benefits over glass, ceramics and metals because of its affordability, flexibility of shape and size, ease of engineering, durability, and control over

its optical quality. Polystyrene is easily obtained at a minimal cost, it can be molded into almost any shape conceivable, and it is durable. All of these benefits make the disclosed method of micro-patterning on glass and plastics affordable, facile, and accurate.

A particular embodiment of the present invention yielding results includes cell patterning on glass and polystyrene using the same simplistic method (see figure 4). Oxygen plasma is used to activate the surface in the case of polystyrene (see figure 2), and acid washing to activate the surface in the case of glass. Both surfaces can be further incubated with a mildly acidic alcoholic solution of aminosilane (see figure 1b) featuring a primary amine on the terminating end of the tethered molecule. Following silanizing, a stencil is applied to the substrates. An aqueous solution of tresyl PEG (see fig 1a) is applied to the substrates around the stencil resulting in regions of exposed amine, and regions of PEG in carefully controlled proximity to one another (see fig 3). After surface modification, the surface can be primed with a cell adhesive protein to speed the cell adhesion process[12].

Materials and Methods:

Reagents and instrumentation that can be utilized in a carrying out the methods of the invention include, but are not limited to, Corning 60 and 35 mm petri dishes cat # 25010, and cat # 25000, VWR micro cover glasses cat # 48368040, Herrick scientific plasma cleaner/sterilizer model PDC-32G, Kurt J Lesker Co. digital convection gauge, trimethoxysilylpropyldiethylenetriamine United Chemical Technologies cat # 35141-30-1, and 2,2,2-trifluoroethanesylphonyl-poly(ethylene)$_{5000}$ glycol Shearwater Polymers cat # M-TRES-5000.

Poly(styrene) substrates are oxygen plasma treated inside a plasma cleaner using the following method. Substrates are placed inside the glass tube chamber and the chamber is evacuated to a pressure of ~200mtorr as indicated by a convection gauge. Oxygen is pulsed in through a regulation valve and the chamber is evacuated again to a pressure of ~200mtorr. The above oxygen pulse is repeated 2 more times. After the last oxygen pulse, the gas is allowed to bleed constantly into the chamber, and the final equilibrium pressure (with the oxygen bleed valve on and the vacuum pump activated) should be ~300mtorr. After the proper pressure is reached, the voltage switch is turned up to HI (100W) and the substrates are treated for 25 min.

Glass surfaces are activated using the following method. Prepare a 1M KOH solution in double DI water. Incubate glass surfaces for 10 min in 1M KOH. After 10 min rinse substrates 3X in double DI water. Soak coverslips in HCl:MeOH (1:1) for 30 min. After the incubation, rinse coverslips in double DI water. Transfer the coverslips into a concentrated bath of sulfuric acid for 30 min, rinse 3x with double DI water. Boil in distilled water for 15 min. Blow the surfaces dry with a nitrogen gun.

Aminosilane treatment is the same for both glass and polystyrene. Prepare a 1% solution of trimethoxysilylpropyldiethylenetriamine in mildly acidified methanol (94% methanol, 5% water, and 0.004% glacial acetic acid). Incubate with substrates for 15 min. Following silanizing, rinse the substrates with methanol and bake in a 80C oven for 30 min.

Apply PDMS stencil to the aminated glass or polystyrene (this embodiment includes but is not limited to 200 micron and 500 micron spots). Apply pressure until PDMS makes a tight seal.

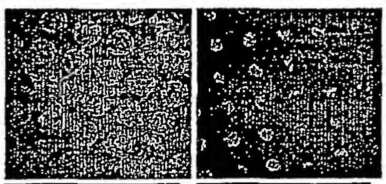
Tresyl-PEG treatment is the same for glass and polystyrene. After stencil application, prepare a 0.12M sodium bicarbonate solution in water. This will be used as the solvent for the tresyl-PEG. Prepare a 19% solution of tresyl-PEG (by weight) in the bicarbonate. Apply the

solution to the stencil and allow the solution to pool around the PDMS resulting in the liquid touching only exposed aminated surface areas. Allow substrates to incubate for 4 hours.

Following PEG treatment, the surfaces are rinsed with the 0.12M sodium bicarbonate solution. After rinsing, the substrates can be coated with fibronectin at a concentration of 25ug/mL PBS. The substrates are allowed to incubate for 2 hours and rinsed under a stream of PBS.

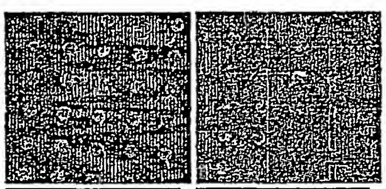
Cells are plated on these substrates after rinsing at a seeding density of 7000cells/cm^2.

Results: 3T3 cells plated on substrates, fixed, permeabilized and stained with a fitc-F-actin stain, images at 2.5x



500 micron spots on plastic

200 micron spots on plastic



200 micron spots on glass

200 micron spots on glass

Discussion and Conclusions:

The present invention provides novel methods for patterning cells on glass and polystyrene substrates. Cell adhesive cues can be defined by the use of a stencil, which has no size constraints. Cell repulsive cues, which also can be defined by the stencil, are tethered to a self- assembled monolayer of an aminosilane. The entire system is coated with a cell adhesive protein and seeded with cells resulting in a micropatterned array of cells. The benign nature of the chemistry employed makes it attractive for biological applications, allows the array on any thermoplastic and thermoset of choice including, but not limited to poly(styrene), PDMS, poly(carbonate), poly(vinyl) chloride, poly(ethylene), poly(ethylene) terapthalate, Teflon, and FEP. The present methods also have the ease and flexibility to be applied to polystyrene and glass substrates using the same method.

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[1-17]

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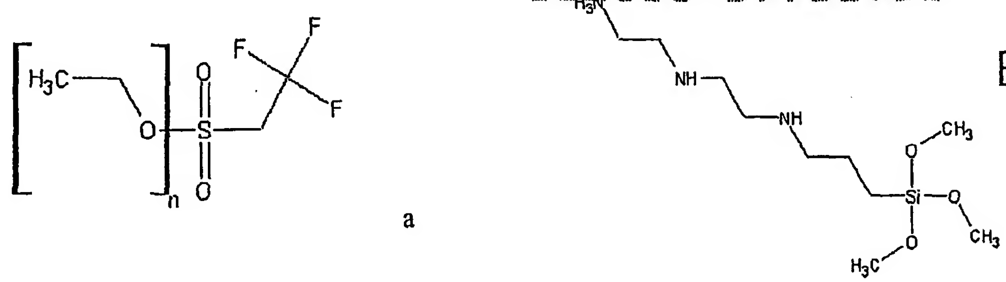
Basic structures and concepts



a b

Figure 1a, the standard structure of tresyl-PEG,
n can equal any number, the data utilizes n = 5000 daltons, figure 1b, structure of trimethoxy silylpropyldiethylenetriamine

a b

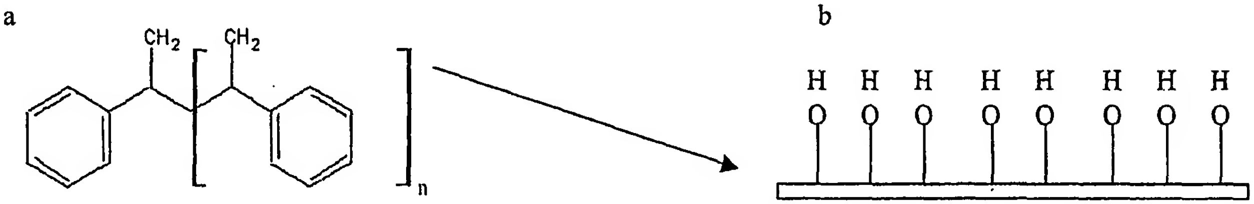


Figure 2, a: the repeat unit of polystyrene b: how the surface may look after oxygen plasma treatment, note: there will be many more oxygen moieties after treatment, but hydroxyl groups are the moiety of interest. Acid clean glass will also resemble 2b

a b c

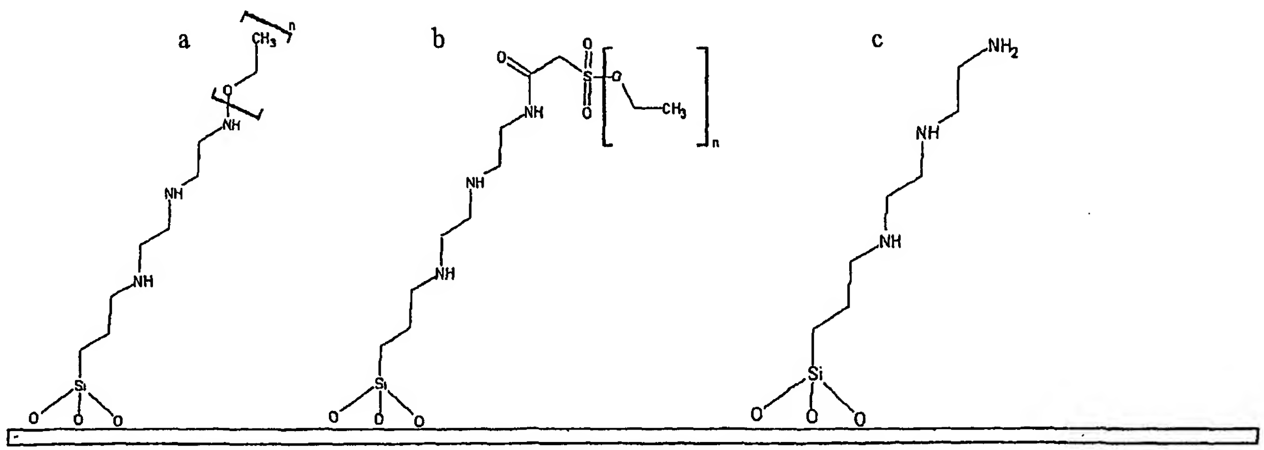


Figure 3, a:amine-PEG surface product, b: sulphonate-amide surface product, c: surface amine

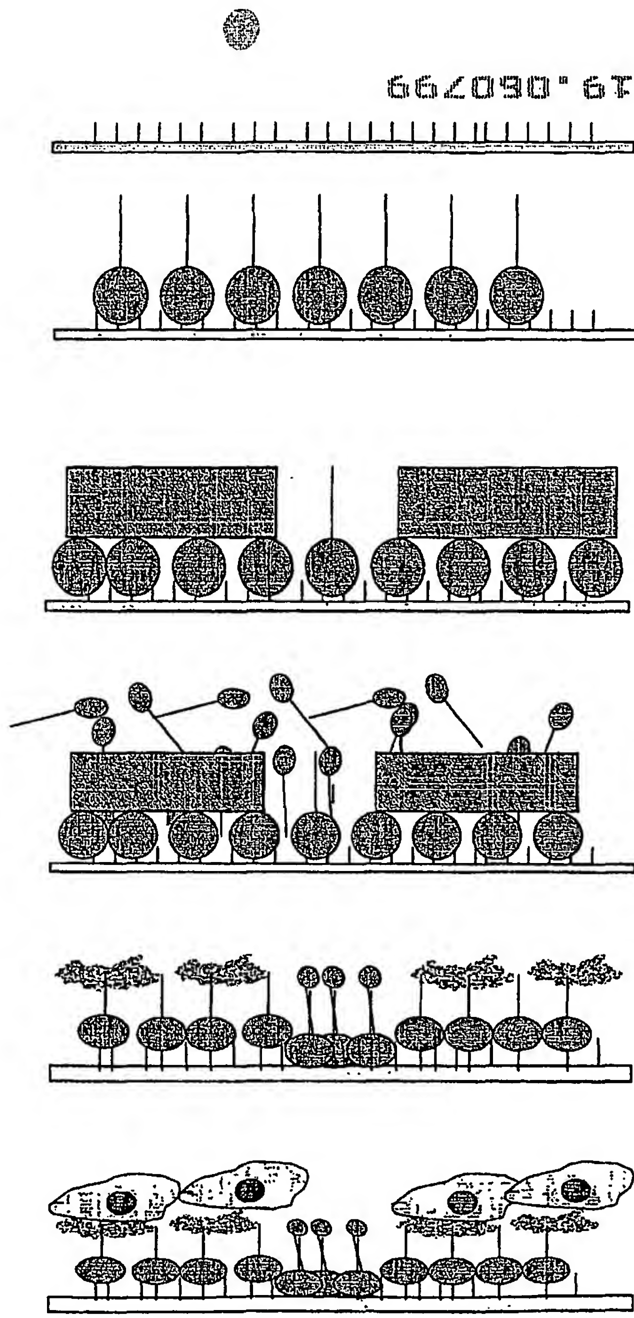


Figure 4, selective positioning of cell adhesive and cell repulsive cues